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Gene expression study in different '*Ca.* Phytoplasma mali'-infected micropropagated *Malus* genotypes

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Abstract

A cDNA-AFLP analysis was carried out on five different *Malus* genotypes, susceptible or resistant towards apple proliferation disease. The gene expression in healthy and '*Candidatus* Phytoplasma mali' infected plants was compared. Established *in vitro* cultures were used to produce standardised '*Ca*. P. mali'-infected plants through *in vitro* grafting. Differentially expressed cDNA-AFLP bands were individuated by the comparison of the pattern obtained for susceptible and resistant genotypes. The cDNA-AFLP fragments were excised from the gel, cloned and sequenced. Apart from genes for which no putative function could be attributed, the identified genes could be grouped into three classes: genes associated with stress response, electron transport or protein degradation and modification. In order to proof their differential expression in the plant-pathogen response a RT-qPCR analysis using the SYBR green II® dye was employed for a first set of sequences.

Key words: Apple proliferation, cDNA-AFLP, real-time RT-qPCR.

Introduction

Apple proliferation (AP) is the most important phytoplasma-associated disease affecting apple. The failure in controlling this disease by standard means strongly increased the importance of resistant genotypes. However, little is known about the resistance mechanisms and the genes involved in the phytoplasma-plant host interaction. The objective of this study was therefore to gain better knowledge about these interactions on the genetic level.

Resistance to AP was discovered mainly in the wild apomictic *Malus* species *Malus sieboldii* and *M. sieboldii*-derived hybrids (Kartte and Seemüller, 1991). Currently, these AP-resistant genotypes were crossed with *M. domestica* genotypes in an ongoing breeding program to obtain AP-resistant apple rootstocks of agronomic value (Jarausch *et al.*, 2007). To elucidate the resistance mechanism a systematic study of the plant-pathogen interaction was started in parallel. As these interactions are difficult to study in woody species *in vivo* where the colonisation with the phytoplasma undergoes seasonal fluctuations, an *in vitro* system was employed.

As previously described, '*Ca*. P. mali' can easily be maintained in micropropagated apple (Jarausch *et al*., 1996) and can be transmitted to healthy apple through *in vitro* grafting (Jarausch *et al.,* 1999). Therefore, the *in vitro* system was also used to establish an *in vitro* resistance screening (Bisognin *et al.,* 2007). By this technique standardised infected material of susceptible and resistant genotypes could be produced which is valuable material to study the gene expression under homogenous and defined conditions. A cDNA-AFLP approach was used to investigate the gene expression after infection of *in vitro* plants belonging to the *Malus domestica* species (AP-susceptible) compared to the *Malus sieboldii* and *Malus sieboldii* x *M. domestica*-derived genotypes (AP-resistant).

Materials and methods

The *in vitro* plants used derived from previously established healthy cultures of Golden Delicious, M9 and the apomictic resistant genotypes *M. sieboldii* and its hybrids H0909 and D2212 (Bisognin *et al.*, 2007). The preparation of the infected material through micrografting was carried out as previously described (Jarausch *et al.*, 1999; Bisognin *et al.,* 2007). Healthy and infected *in vitro* plants were acclimatized *ex vitro* according to standard protocols in spring, at least 12 m.p.i.. For every genotype 6-7 healthy and infected *in vitro* plants were pooled, respectively. Leaves and roots of healthy and infected *ex vitro* plants were collected and pooled from 3 plants of each genotype, respectively. Total RNA extraction from *in vitro* and *ex vitro* material was performed as described by Moser *et al.* (2004). A cDNA-AFLP analysis was performed on the total RNA extracted from the *in vitro* plants as described by Breyne *et al.* (2003). The analysis was carried out for each genotype on both the infected and healthy state using the 32 possible selective primer combinations. Rom the comparison of the cDNA-AFLP patterns those bands that resulted over expressed in the infected resistant genotypes were isolated and cloned using the USER friendly PCR cloning kit (New England Biolabs). The sequences obtained from the clones were analysed by nucleotide-nucleotide BLAST on the EST database present at the NCBI. The development of specific primers was done using the online algorithm Primer3 (frodo.wi.mit.edu). The real-time reaction was performed using the RT-qPCR SYBR Green II kit (Invitrogen) as described by the manufacturer. The PCR and the real-time detection were carried out in a Chromo4 thermal cycler (MJ-Biorad).

Results

The *in vitro* system established and described in Bisognin *et al*. (2007) was used for the production of a set of standardised infected plants from AP-susceptible genotypes (*M. domestica* cvs. Golden Delicious and M9) and from AP-resistant genotypes (*M. sieboldii* and its hybrids H0909 and D2212). For each genotype the cDNA-AFLP procedure was carried out on the RNA extracted from the healthy and the infected plants using 32 primer combinations. The expression pattern obtained from each primer combination was compared between healthy and infected states and between genotypes. More than 400 bands were individuated as putatively differentially expressed. In order to reduce the work required to confirm them only the cDNA-AFLP fragments showing an over-expression in the infected resistant genotypes were selected. About 60 bands were isolated and cloned. Sequencing of a subset of the clones yielded 44 different sequences. The further analysis was focused on those sequences that were produced from the same type of cloned cDNA-AFLP fragment found in more than one resistant genotype. A total of 11 unique sequences were obtained. They were grouped according to the putative function of the corresponding gene in 4 major classes. 39% of the putative genes were associated with stress response; 23% with proteins involved in the electron transport; 22% were genes of unknown function and 16% of the genes were putatively involved in reactions of protein degradation and modification.

A real-time PCR assay was employed to verify if these genes were effectively involved in the plant-pathogen interaction. For this, supplementary to the *in vitro* plants used for cDNA-AFLP, healthy and infected *ex vitro* plants originating from the same cultures were tested. Total RNA was extracted from leaves and roots. Specific primers developed on the 11 sequences were then used in the real-time analysis carried out directly on the total RNA using a RT-qPCR kit with SYBR green II as detection method. Among the selected genes only one gene with unknown function resulted significantly differentially expressed. The other genes were not differentially expressed or not significantly differentially expressed.

Discussion

The results presented here were obtained from the analysis of a first subset of cDNA-AFLP bands isolated after the screening between infected and healthy plants and comparing susceptible and resistant genotypes. The sequence data obtained from these cDNA fragments revealed that genes representing a broad range of metabolisms were found. Although further verification is needed to proof the involvement of these genes in the specific plant response towards '*Ca*. P. mali' the high percentage of genes associated with stress response reactions indicate that the phytoplasma infection induces a multitude of plant defence reactions. The real-time PCR assay was successfully applied to study the gene expression directly on total RNA extracts obtained from *in vitro* as well as from *ex vitro* plants. At least one gene could be shown to be differentially expressed *in vitro* and *ex vitro* indicating that the results obtained *in vitro* are transferable to the *in vivo* conditions. Unfortunately, the function of this gene is unknown.

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References

BISOGNIN C., CICCOTTI A. M., MOSER M., GRANDO M .S., JARAUSCH W., 2007.- Establishment of an in vitro screening system of apple proliferation-resistant rootstocks genotypes based on micrografting.- *Acta Horticolturae*, in press.

BREYNE P., DREESEN R., CANNOOT B., ROMBAUT D., VANDEPOELE K., ROMBAUTS S., VANDERHAEGHEN R., INZE´ D., ZABEAU M., 2003.- Quantitative cDNA-AFLP analysis for genome-wide expression studies.- *Molecular Genetics and Genomics,* 269: 173-179.

JARAUSCH W., LANSAC M., DOSBA F., 1996.- Long-term maintenance of non-culturable apple proliferation phytoplasmas in their micropropagated natural host plant.- *Plant Pathology,* 45: 778-786.

JARAUSCH W., LANSAC M., BLIOT C., DOSBA F., 1999.- Phytoplasma transmission by in vitro graft inoculation as a basis for a preliminary screening method for resistance in fruit trees.- *Plant Pathology,* 48: 283-287.

JARAUSCH W., BISOGNIN C., PECCERELLA T., SCHNEIDER B. SEEMÜLLER E., 2007.- Development of resistant rootstocks for the control of apple proliferation disease.- *Acta Horticulturae*, in press.

KARTTE S., SEEMÜLLER E., 1991.- Susceptibility of grafted *Malus* taxa and hybrids to apple proliferation disease.- *Journal of Phytopathology*, 131: 137-148.

MOSER C., GATTO P., MOSER M., PINDO M., VELASCO R., 2004.- Isolation of functional RNA from small amounts of different grape and apple tissues.- *Molecular Biotechnology*, 26 (2): 95-100.

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